THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

Grant #869

Application for Research Grant
(Use extra pages as needed)

Date: 1/2/75

1. Principal Investigator (give title and degrees):
Ronald P. Rubin, Ph.D., Professor of Pharmacology and Chief, Autonomic-Cardiovascular Division

2. Institution & address:
Virginia Commonwealth University
Medical College of Virginia
12th & Clay Streets
Richmond, Virginia 23298

3. Department(s) where research will be done or collaboration provided:
Department of Pharmacology

4. Short title of study:
The Action of Nicotine on the Adrenal Gland

5. Proposed starting date: July 1, 1975

6. Estimated time to complete: One to two years

7. Brief description of specific research aims:
Our previous investigations on this project have shown that nicotine enhances basal corticosteroid release and potentiates the steroidogenic effects of adrenocorticotropin (ACTH) on isolated trypsin dispersed cat adrenocortical cells. The details concerning the intimate mechanisms involved in nicotine's action as a secretogogue are still incompletely understood. Insight into the nature of the factors governing its stimulatory effect on the adrenal cortex is of critical importance in gaining a clearer understanding of the molecular processes underlying the fundamental process of secretion. Moreover, knowledge of the effects of nicotine on the gland which plays such significance and importance in light of the widespread use and diverse pharmacologic effects of this alkaloid.

Thus, the primary aim of this ongoing investigation is to ascertain the nature of the facilitatory action of nicotine on adrenocortical secretion, through a comprehensive study of the relative roles played by calcium, cyclic AMP, and prostaglandins. It is our feeling that the results of this project may lead to a better understanding of some of the health-related problems associated with tobacco smoke. A better understanding of these problems could lead to a more rational approach to their solution.

8. Brief statement of working hypothesis:

Calcium, cyclic AMP, and prostaglandins have all been implicated in the sequence of events leading to corticosteroid synthesis and release. Therefore, a key to understanding the steroidogenic action of nicotine involves a comprehensive investigation of the possible role(s) played by these putative mediators.

9. Details of experimental design and procedures (append extra pages as necessary)

Structure-Activity Studies. In our continuing investigation on the mechanism of nicotine action as a secretogogue, we recently developed the technique for isolating cat adrenocortical cells by tissue dispersion with trypsin, which enables us to study responses of homogeneous populations of intact cortical cells (see accompanying manuscript by Rubin and Warner for details). The ability of nicotine to augment steroidogenesis in this cortical cell preparation raises the question as to the specificity of this effect. Therefore, experiments are planned to compare the steroidogenic activity of metabolites of nicotine, such as nornicotine and cotinine, as well as related compounds such as lobeline. Such studies will ascertain whether the ability of nicotine to enhance steroidogenesis can be related to its well-defined pharmacologic activity in other biological systems.

Cyclic Nucleotide Studies. Putative mediators of hormone and drug action such as calcium, cyclic AMP, and prostaglandins have been the focus of intensive scientific inquiry over the past several years. Since our previous investigations on this isolated cortical cell preparation have demonstrated that the steroidogenic activity of nicotine is obtunded by calcium deprivation and enhanced by dibutyryl cyclic AMP and by prostaglandin (PGE_2) (Rubin and Warner), follow-up studies are indicated to ascertain whether the action of nicotine involves alterations in the cellular levels of cyclic AMP or prostaglandin.

Preliminary experiments indicate that steroidogenic concentrations of nicotine, like those of ACTH, enhance cyclic AMP levels in isolated cortical cell preparations. It is not clear what relationship, if any, this increase in cyclic AMP has to the process by which nicotine evokes steroid release, since other steroidogenic agents such as the calcium

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9. Details of experimental design and procedures (continued)

ionophore, A-23187, and PGE_2 are unable to significantly elevate cyclic AMP levels, at least in the concentrations so far tested. A more comprehensive investigation -- including exposure to various concentrations of these stimulating agents for different time intervals -- is required before more definitive conclusions concerning the role of cyclic AMP in the steroidogenic activity of nicotine, and other substances, can be derived.

Cyclic GMP is another cyclic nucleotide which is ubiquitously distributed in biological systems and is thought to exert effects which are counter to those of cyclic AMP (Goldberg et al., 1973). This idea of biological "dualism" implies that a cellular event which opposes that produced by an increase in cyclic AMP might be produced by an elevation of tissue cyclic GMP rather than by a "passive" decrease in cyclic AMP. It is therefore of interest to study the effects of nicotine on cortical levels of cyclic GMP. Isolated cat adrenocortical cells will be exposed to graded nicotine concentrations for various time intervals (up to four hours). The cells will be centrifuged and the pellet extracted with trichloroacetic acid. Following centrifugation the supernatant is applied to a AG1X8 column (Bio-Rad) and eluted with perchloric acid to separate cyclic GMP and cyclic AMP. The samples are then lyophilized, diluted with buffer, and assayed for either cyclic AMP or GMP by radioimmunoassay. This technique employs antibodies generated against succinyl-cyclic AMP or succinyl-cyclic GMP (Steiner et al., 1969).

Prostaglandin Studies. Prostaglandins are a group of unsaturated fatty acids which are ubiquitously distributed in tissues and are thought to play a role in endocrine secretory mechanisms (Flack, 1973). Experiments of similar design will be employed to study the effects of nicotine on prostaglandin levels in adrenocortical cells. Antibodies to $PGF_{1\alpha}$ and $PGF_{2\alpha}$ have been made in our laboratory by injecting rabbits with $PGF_{1\alpha}$ or $PGF_{2\alpha}$ which are covalently linked to serum albumin by reaction with carbodiimide reagent. The antisera obtained from these rabbits have been successfully used for determination of tissue $PGF_{1\alpha}$ and $PGF_{2\alpha}$ by radioimmunoassay. Thus, prostaglandin determinations of adrenocortical cells exposed to various nicotine and ACTH concentrations for different time intervals will be carried out. Separation of the various PG's on silicic acid columns precedes the radioimmunoassay procedure. Just as with the cyclic nucleotide studies, an attempt will be made, if possible, to establish a correlation

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9. Details of experimental design and procedures (continued)

between prostaglandin levels and the steroidogenic response. Theoretically, such a correlation could be made either quantitatively and/or temporally.

Calcium Studies. Calcium is another potential mediator of steroidogenesis, since strong evidence exists to support the concept that this action is a necessary factor in ACTH- and nicotine-induced steroidogenesis (see accompanying manuscript, Rubin and Warner). The critical role of calcium may be related to the fact that it, in some as yet unknown manner, permits specific alterations in levels of cyclic nucleotide or prostaglandin which follow stimulation by nicotine. Therefore, the effects of nicotine on cyclic nucleotide and prostaglandin levels will be studied in cortical cells incubated in calcium-deprived media, as well as in calcium-containing media. Experiments are also planned to study the effects of ACTH and nicotine on radiolabeled (^{45}Ca) fluxes, to ascertain whether the actions of these agents are associated with a cellular redistribution of calcium. A characterization of the relationships between calcium, cyclic nucleotides and prostaglandins should contribute greatly to our understanding of the fundamental mechanism whereby nicotine stimulates hormone secretion.

Studies with Radioactive Nicotine. In our soon-to-be published article in the British Journal of Pharmacology, the question was raised as to whether nicotine stimulates steroidogenesis by an action on the surface of cortical cell membrane or whether it must penetrate the cell to elicit its effects. Such information is, of course, critical for ascertaining nicotine's mode of action. Thus, the uptake and accumulation of radioactive (^{3}H or ^{14}C) nicotine in isolated cat adrenocortical cells will be studied over various time intervals, in order to determine whether a relationship exists between nicotine entry into cortical cells and its steroidogenic activity. Cells will be exposed to labeled nicotine for various time intervals and measurement of the radioactive nicotine space carried out. This space is defined as: cpm/gm tissue:cpm/ml of medium. Alternatively, after exposure to nicotine, the cells will be returned to non-radioactive medium and washout of the labeled alkaloid instituted. From derived efflux curves an estimation can be made of the cellular distribution of nicotine. A significant accumulation of nicotine in cortical cells would necessitate additional chemical studies to ascertain whether the alkaloid was present as unchanged nicotine or as its major metabolite, cotinine.

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9. Details of experimental design and procedures (continued)

The general significance of this proposed investigation lies in the fact that the physiological actions of adrenocortical hormones are observed in most tissues and are vital for the normal functioning of many organs and for their responses to stress. In addition, metabolic aberrations which appear during pregnancy, such as toxemia and the diabetogenic state may be related to the increased adrenal function which accompanies gestation. Thus, an agent such as nicotine which can affect steroid hormone production and release will have marked effects on a variety of bodily functions. For example, since adrenal steroids are potent immunosuppressives, the stimulatory effects of nicotine on the cortex may be responsible for the impaired immunological response observed in chronic cigarette smokers (Thomas et al., 1974). Accordingly, in light of the widespread use of nicotine, it is of considerable interest and importance to have a basic understanding of the mechanism whereby this alkaloid elevates steroid levels.

10. Space and facilities available (when elsewhere than item 2 indicates, state location):

The principal investigator, as Professor of Pharmacology and head of the Cardiovascular and Autonomic Division, has available approximately 2400 square feet of laboratory and office space. The laboratories are well-equipped and house both beta and gamma counting instruments, tissue incubators, automatic pipeting devices, and refrigerated centrifuges. In addition, the principal investigator has access to a well-equipped animal facility run directly by the Department of Pharmacology.

The Thompson-McCaw library, directly across the street from the Pharmacology Department, contains a complete collection of books and journals. The library has direct access to the SUNY and N.L.M. computer search services.

MCV has full computer center facilities and these services are directly available to the project. The investigator's laboratory also has a Hewlett-Packard (Model 9810) programmable calculator for more routine calculations.

The Department has a small shop and a trained mechanic, and the Medical College has fully-equipped mechanical, instrument and electronic shops which are available through the Department of Biophysics.

11. Additional facilities required:

None

12. Biographical sketches of investigator(s) and other professional personnel (append):

see enclosed curriculum vitae.

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

see enclosed curriculum vitae.

14. First year budget:

A. Salaries (give names or state "to be recruited") Professional (give % time of investigator(s) even if no salary requested)	% time	Amount
Ronald P. Rubin (Principal Investigator)	40	---
Technical:		
Research Assistant	100	REDACTED
*includes 11% for fringe benefits		
		REDACTED
Sub-Total for A		

B. Consumable supplies (by major categories)

Glassware	$ 1,000
Radioactive Chemicals	1,000
Animals	1,000
Other Miscellaneous Lab Items	1,000
Sub-Total for B	$ 4,000

C. Other expenses (itemize)

Travel (to attend scientific meetings)	
Sub-Total for C	$ 500
Running Total of A + B + C	R

D. Permanent equipment (itemize)

Sub-Total for D	-0-
E. Indirect costs (15% of A+B+C)	E 2,475
15. Estimated future requirements:	Total request $18,975

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	R	4,000	500	--	2,475	18,975
Year 3		4,000	500	--	2,475	18,975

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16. Other sources of financial support:
List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
The Action of Calcium on Secretion	AM 18066 PHRA	52,407	6/1/74 - 2/28/77

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
None			

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Ronald P. Rubin, Ph.D.

Signature Ronald P. Rubin Date 1/5/75

Telephone R
Area Code Number Extension

Check's payable to:
Mr. Raymond Holmes, Jr.
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Responsible officer of institution

Typed Name Dr. John J. Salley

Title Assoc. Vice Pres for Research & Graduate Affairs

Signature John J. Salley Date 1/9/75

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Summary Progress Report

CTR Grant #869

Progress Report #2
6/1/74 - 1/1/75

Name of Investigator: Ronald P. Rubin, Ph.D.

Name of Institution: Virginia Commonwealth University
Medical College of Virginia

Mailing Address: Department of Pharmacology (Box 726)
Medical College of Virginia
Richmond, Virginia 23298

Title of Grant: The Action of Nicotine on the Adrenal Gland

During the past year and a half our laboratory has been engaged in a comprehensive study of the stimulatory effects of nicotine on the cat adrenal gland. The major thrust of this investigation has been from two major perspectives: (a) to elucidate the role of cyclic AMP in the mechanism whereby nicotine, acetylcholine and other medullary secretogogues enhance catecholamine secretion and (b) to discern whether nicotine exerts a direct action on adrenocortical cells, and if so, by what mechanism. Since the details of this work are elaborated and conclusions amply documented in the accompanying reprint (Jaanus and Rubin, 1974) and preprint (Rubin and Warner, in press), only the highlights of these studies will be presented here.

(a) Medulla. Earlier work established that calcium is an absolute requirement for nicotine and acetylcholine-induced release of catecholamines by the cat adrenal medulla (Douglas and Rubin, 1961b). Since cyclic AMP has been implicated as a "second messenger" in many physiological and pharmacological responses (Robison et al., 1971), it is well understood that the elucidation of the nature of calcium action in nicotine-induced catecholamine release requires intimate knowledge of the role of cyclic AMP. Therefore, in collaboration with Dr. Siret Jaanus (Jaanus and Rubin, 1974), the effects of nicotine on cyclic AMP levels and catecholamine release were determined in cat adrenal glands perfused in situ with Locke's solution.

Initially, cyclic AMP analyses (method of Steiner et al., 1969) were carried out on the cat medulla and cortex, after separating these two organs. The mean basal levels in medulla and cortex were 30 ± 13 and 172 ± 6 pmoles/gland, respectively. Thus, establishing the presence of cyclic AMP in the medulla, experiments were carried out to discern if a quantitative and/or temporal correlation existed between medullary cyclic AMP levels and catecholamine secretion following stimulation by nicotine. Indeed, perfusion with nicotine or acetylcholine (4×10^{-5} g/ml) elevated adrenal cyclic AMP levels and enhanced its rate of release into the adrenal perfusate. However, the time course of the changes in tissue cyclic AMP during stimulation was out of phase with the time course of catecholamine release. Maximal increases in cyclic AMP were not manifest until after eight minutes of exposure to the secretogogue, whereas maximal rates of secretion occurred during the first minute. The lack of correlation between adrenal cyclic AMP levels and catecholamine release was also reflected in experiments with theophylline, an inhibitor of phosphodiesterase. Theophylline, in concentrations which augment adrenal cyclic AMP levels, failed to enhance basal catecholamine release and did not potentiate the secretory response to a submaximal concentration of nicotine.

These data are interpreted to mean that the elevation of secretion effected by nicotine is not directly monitored by adrenal cyclic AMP levels in a manner which resembles that of calcium. These findings do not mitigate the possibility, however, that one or more alternate mediators are interposed between the response triggered by nicotine and the release of hormone. Therefore, experiments are now in progress to determine whether the stimulatory action of nicotine on the adrenal medulla is better correlated with alterations in cyclic GMP and/or prostaglandin levels.

Cortex. Up to now, controversy has existed as to whether nicotine is able to elevate corticosteroid secretion by a direct action on adrenocortical cells (Kershbaum et al., 1968; Suzuki et al., 1973). Our recent work has clearly established (see preprint) that nicotine possesses the potentiality of directly stimulating isolated cat adrenocortical cells. The stimulant action manifests itself in micromolar concentrations, is dose-dependent and like the physiological stimulus, ACTH, depends upon the presence of calcium. Not only is nicotine able to stimulate cortical cells directly but it enhances the steroidogenic effect of ACTH in an additive, rather than synergistic, manner.

The further observations that nicotine enhances the steroidogenic action of exogenous cyclic nucleotide and prostaglandin E_2, just as it enhances the action of ACTH, suggest that these proposed mediators of steroidogenesis play crucial roles in mediating the action of nicotine. However, support of such an hypothesis requires the measurement of changes in tissue levels of these putative mediators during stimulation by nicotine, and these experiments are now in progress.

The role of calcium in the steroidogenic action of nicotine also cannot be defined at present. Nicotine-like acetylcholine stimulates the medulla to secrete by depolarizing the chromaffin cell membrane (Douglas et al., 1967), facilitating transmembrane calcium flux, which in turn triggers the release of preformed catecholamine stored in secretory granules (see Rubin, 1970). On the other hand, in the cortex there appears to be no correlation between steroidogenic activity and the depolarization of cortical cells (Jaanus et al., 1970), and stimulation by ACTH results from an intracellular translocation of calcium (Jaanus and Rubin, 1971). Since nicotine can traverse cell membranes and release calcium from cellular binding sites (Weiss, 1968) an intracellular mobilization of calcium may be responsible for nicotine-induced steroidogenesis. Experiments are now in progress to compare the effects of ACTH and nicotine on radiocalcium fluxes in the isolated cat adrenocortical preparation. Such studies will provide salient information as to whether the action of nicotine on the adrenal cortex is primarily associated with a transmembrane flux of extracellular calcium or a redistribution of intracellular calcium.

Although our studies have uncovered valuable information concerning the mode of action of nicotine on adrenal hormone release, we still have an incomplete picture of the processes involved in this action. Despite the recognized importance of nicotine, as a result of its widespread use, there have been surprisingly few experimental studies concerned with the mechanism by which this pharmacologic agent affects non-neuronal endocrine cells. There is therefore an obvious gap in our knowledge regarding cellular events occurring during nicotine-induced activation of the secretory process. Advances made in our knowledge of the intimate mechanism of nicotine action by a continuation of this study would have important implications and immense value in regard to understanding and treating the pathophysiological states related to the chronic use of tobacco.

References alluded to in the sections on experimental design and in the Summary Progress Report

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Rubin, R.P. [illegible]

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CURRICULUM VITAE
Ronald P Rubin

Date & Place of Birth: REDACTED

Marital Status: REDACTED

Education:

Harvard University	A.B. (Biochemistry)	1954
Attended Harvard School of Dental Medicine		1954-57
Harvard Graduate School of Education	A.M.T. (Science Ed.)	1958
Albert Einstein College of Medicine	Ph.D. (Pharmacology)	1963

Positions:

1974-present	Professor & Chief, Autonomic-Cardiovascular Division, Department of Pharmacology; Medical College of Virginia
1970-1974	Associate Professor, Department of Pharmacology; State University of New York, Downstate Medical Center
1966-1970	Assistant Professor, Department of Pharmacology; State University of New York; Downstate Medical Center
1964-1966	Instructor, Department of Pharmacology; State University of New York; Downstate Medical Center
February 1964-June 1964	Instructor, Department of Physiology; Hunter College; New York City (Evenings)
1963-1964	Postdoctoral Fellow, Department of Pharmacology; Albert Einstein College of Medicine
1958-1959	Science Instructor, White Plains High School; White Plains, New York

Society Memberships:

REDACTED

Committee Memberships:

Committee of the Faculty, 1972; Medical School Admissions Committee, 1972 (Downstate Medical Center); FASEB Public Information Committee for ASPET (1972-1975)

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1. Rubin, R.P. The role of calcium in the release of neurotransmitter substances and hormones. Pharmacol. Rev. 22: 389-428 (1970).

2. Jaanus, S.D. and Rubin, R.P. The perfused adrenal gland: In Hormones and Cyclic Nucleotides (a volume of Methods in Enzymology): Submitted for publication.

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2. Douglas, W.W. and Rubin, R.P. The role of calcium in adrenal medullary secretion evoked by acetylcholine or potassium. J. Physiol. 159: 24-25P (1961).

3. Douglas, W.W., Poisner, A.M. and Rubin, R.P. Stimulus-secretion coupling at the adrenal medulla. XXII Int. Cong. of Physiol. Sciences, Leiden, Excerpta Medica, Int. Cong. Series 48: 827 (1962).

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